EVALUATION OF LACCASES PRODUCED BY *Cyathus bulleri* ON WHEAT BRAN

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by

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Submitted

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This research work is dedicated to my beloved family for their unconditional love and support……

CERTIFICATE

This is to certify that the thesis entitled **"Evaluation of laccases produced by *Cyathus bulleri* on Wheat Bran"** being submitted by **Ms. Arpita Vats** to the Indian Institute of Technology Delhi, for the award of the degree of **'Doctor of Philosophy'**, is a record of the bonafide researchwork carried out by her, which has been prepared under my supervision and guidance in conformity with the rules and regulations of the Indian Institute of Technology, Delhi. The research reports and the results presented in this thesis have not been submitted for any degree or diploma in any other University or Institute.

Prof. Saroj Mishra
Professor
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Date:

Place:

Acknowledgements

PhD is a long journey spanning 5 years, comprising of both lows and highs. There were eureka moments and at the same time, disheartening phases when things fell apart. Without this bunch of people, I am acknowledging here, it would have been impossible for me to witness this day.

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ABSTRACT

Textile industry is one of the major polluting sources, adding toxic and carcinogenic dyes to the water bodies, thereby depleting the water quality. In an attempt to look for an efficient enzyme based approach for the treatment of dye-containing textile effluents, *Cyathus bulleri*, a white rot fungus, was cultivated on low-cost agro-residues, namely, wheat bran (WB), wheat straw (WS) and domestic waste orange peel (OP) in semi-solid fermentation for production of ligninolytic enzymes. Of a large number of enzymes that are likely to be involved, activities of laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) were investigated on these substrates. Of the three substrates, WB and OP supported maximum laccase production (specific activity of 679.6 U g^{-1} WB and 579.8 U g^{-1} OP) while activities of MnP and LiP were found to be low. Most importantly, no supplements, metal salts or aromatics were required for laccase production. Culture filtrates obtained on WB and OP were found to be extremely efficient in decolorization (65-85%) of synthetic dyes. New finding was high decolorization of Kiton blue A and Reactive orange 16, the products of which were identified by electron spray ionization-mass spectrometry (ESI-MS) and pathways of degradation proposed. WB-produced culture filtrate also decolorized and detoxified real and simulated textile effluents by about 40%, the former not requiring addition of any mediator

Laccase was purified from the culture filtrate of WB grown fungus and characterized. In comparison to basal salt medium (BSM) grown fungus, the molecular weight of the laccase from WB grown fungus was slightly lower (by ~5 kDa) and it displayed higher tolerance towards organic solvents, EDTA and NaCl. It also decolorized diverse categories of dyes (azo, triarylmethane, phthalocyanine, anthraquinone) by 65-95%. The resulting metabolites of dyes were identified using ESI-MS and the degradation pathways of several

dyes (many of them, for the first time) proposed. The WB purified laccase also degraded textile effluent more efficiently (~57%) compared to BSM purified laccase (~20%). The most distinguishing feature of this laccase was increased oxidation of non-phenolic veratryl alcohol and reactive blue 21 without assistance of any mediator. The unique properties of this laccase were attributed to presence of various isoforms, as determined by *de novo* sequencing of tryptic fragments.

Transcriptome sequencing of the WB grown fungus indicated presence of 8 laccase isoforms along with 5 isoforms of MnP. The sequences of 4 of the laccase isoforms were successfully mapped back to the peptide data obtained from *de-novo* sequencing. The identified laccase isoforms were theoretically characterized with the help of available softwares and phylogenetic trees constructed. Various isoforms displayed good protein homology (identity $\geq$ 50%) among each other and with other known fungal laccases. Amino acid sequence alignment showed dissimilar regions at the N- and C-terminus. Modelled structures of laccase isoforms were compared and these were found to differ significantly in the nature of the amino acid residues located in the vicinity of copper atoms at the catalytic site, which may contribute to the difference in substrate specificity of each isoform. Along with ligninolytic enzymes, a total of 738 carbohydrate active enzymes were predicted which were further classified into sub-classes and families. Also, a total of 491 plant cell wall degrading enzymes were identified and categorized into cellulose, hemicelluloses, lignin and pectin degrading groups.

सार

वस्त्र उद्योग प्रमुख प्रदूषण स्रोतों में से एक है, जिसके जल निकासों में आमतौर पर जहरीले और कैंसरजन्य रंग मौजूद होते हैं, जिनसे पानी की गुणवत्ता कम हो जाती है। डाई युक्त प्रदूषित पानी के उपचार के लिए एक कुशल एंज़ाइम आधारित दृष्टिकोण की तलाश करने के प्रयास में, साइथस बुलरी, एक सफेद रॉट फंगस को कम लागत वाले कृषि अवशेषों, अर्थात् गेहूं की चोटी (डब्ल्यूबी), गेहूं के भूसे (डब्ल्यूएस) और घरेलू अपशिष्ट नारंगी छील (ओपी) पर लिग्निन नाशक एंजाइमों के उत्पादन के लिए सेमि सॉलिड फेरमेंटशन स्तिथि में उपजाया गया । इन सबस्ट्रेट्स पर उत्पादित एन्ज़ाइमों की एक बड़ी संभावित संख्या में से लैक्केस, मैंगनीज पेरोक्सिडेस (एमएनपी) और लिग्निन पेरोक्सिडेस (लीप) की एक्टिविटीज़ की जांच की गई। तीन सब्सट्रेट्स में, डब्लूबी और ओपी ने अधिकतम लैक्केस उत्पादन (679.6 यू प्रति ग्रा डब्ल्यूबी और 57 9.8 यू प्रति ग्रा ओपी की स्पेसिफिक एक्टिविटी) का समर्थन किया, जबकि एमएनपी और लीप की एक्टिविटीज़ कम पायी गयीं। सबसे महत्वपूर्ण बात यह है कि लैक्केस उत्पादन के लिए कोई पूरक, मेटल साल्ट्स या अरोमैटिक्स की आवश्यकता नहीं थी। डब्लूबी और ओपी पर प्राप्त कल्चर फिल्ट्रेटस सिंथेटिक रंगों के विकृतिकरण (65-85%) में बेहद कुशल पाए गए। नई खोज किटोन ब्लू ए और रिएक्टिव ऑरेंज 16 का उच्च विकृतिकरण था, जिसके अंत उत्पादों को इलेक्ट्रॉन स्प्रे आयनीकरण-द्रव्यमान स्पेक्ट्रोमेट्री (ईएसआई-एमएस) द्वारा पहचाना गया और विघटित मार्गों को प्रस्तावित किया गया। डब्लूबी-उत्पादित कल्चर फिल्ट्रेट ने लगभग 40% तक वास्तविक और सिमुलेटेड कपड़ा अपशिष्ट जल को विघटित और डिटॉक्सिफाइ किया, जिसमें किसी भी रासायनिक मध्यस्थ को मिलाने की आवश्यकता नहीं पायी गयी।

डब्लूबी पर उत्पादित लैक्केस को कल्चर फिल्ट्रेट से प्योरिफाई एवं कैरैक्टराइज़ किया गया। बेसल साल्ट माध्यम (बीएसएम) पर उत्पादित लैक्केस की तुलना में, डब्ल्यूबी पर उत्पादित लैक्केस का आणविक भार थोड़ा कम था (~ 5 केडीए) और उसने कार्बनिक सॉल्वेंट्स, ईडीटीए और सोडियम क्लोराइड की ओर उच्च सहनशीलता प्रदर्शित की। इसने रंगों की विविध श्रेणियों (एज़ो, ट्रायरेल्मेथेन,

थायोसाइनिन, एंथ्राक्विनोन) को 65-95% तक भी विघटित किया। रंगों के परिणामी मेटाबोलाइट्स को ईएसआई-एमएस का उपयोग करके और कई रंगों के अवक्रमण मार्ग (उनमें से कई, पहली बार प्रस्तावित) का उपयोग करके पहचाना गया था। डब्लूबी शुद्ध लैक्केस ने बीएसएम शुद्ध लैक्केस (~ 20%) की तुलना में कपड़ा अपशिष्ट जल को अधिक कुशलतापूर्वक (~ 57%) विघटित किया। इस लैक्केस की सबसे विशिष्ट विशेषता किसी भी रासायनिक मध्यस्थ की सहायता के बिना गैर-फेनोलिक वेरेट्राइल अल्कोहल और रिएक्टिव ब्लू 21 के ऑक्सीडेशन में वृद्धि होना था। इस लैक्केस के अनूठे गुणों का कारण विभिन्न आइसोफॉर्म की उपस्थिति पाया गया, जो कि ट्रिप्टिक पेप्टाइड के डी-नोवो सिक्वेंसिंग द्वारा निर्धारित किया गया।

डब्ल्यूबी पर उगाए गए साइथस बुलरी के ट्रांसक्रिप्टोम सिक्वेंसिंग ने एमएनपी के 5 आइसोफॉर्म के साथ 8 लैक्केस आइसोफॉर्म की उपस्थिति का संकेत दिया। लैक्केस आइसोफॉर्म के 4 अनुक्रमों को डी-एनवो सिक्वेंसिंग से प्राप्त पेप्टाइड डेटा पर सफलतापूर्वक मैप किया गया था। पहचाने गए लैक्केस आइसोफॉर्म उपलब्ध सॉफ्टवेयर द्वारा कैरैक्टराइज़ किये गए और फाईलोजेनेटिक ट्री बनाये गए। विभिन्न आइसोफॉर्मस ने एक दूसरे के बीच और अन्य ज्ञात फंगल लैक्केस के साथ अच्छी प्रोटीन अनुरूपता (पहचान $\geq$ 50%) का प्रदर्शन किया। एमिनो एसिड सीक्वेंस एलाइनमेंट ने एन- और सी-टर्मिनस में भिन्न क्षेत्रों को दिखाया। लैक्केस आइसोफॉर्मस की मॉडलिंग संरचनाओं की तुलना की गई और ये एक्टिव साइट पर कॉपर एटमस के आस-पास स्थित एमिनो एसिड अवशेषों की प्रकृति में काफी भिन्न पाए गए थे, जो प्रत्येक आइसोफॉर्म की सब्सट्रेट विशिष्टता में अंतर में योगदान दे सकते हैं। लिग्निनोलिटिक एन्ज़ाइमों के साथ, कुल 738 कार्बोहाइड्रेट सक्रिय एन्ज़ाइम्स प्रस्थापित किये गए जिन्हें विभिन्न श्रेणी और उप- श्रेणी में वर्गीकृत किया गया था। साथ ही, 491 प्लांट सेल वाल विघटित एन्ज़ाइम्स की पहचान की गई और सेलूलोज़, हेमिसेल्यूलोज़, लिग्निन और पेक्टिन अपघटन समूहों में वर्गीकृत किया गया।

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LIST OF ABBREVIATIONS

Abbreviations	Full forms
2-DE	2-Dimentional electrophoresis
BSM	Basal salt medium
ABTS	2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
BG	Basic green 4
BME	β-Mercaptoethanol
BLAST	Basic local alignment tool
BOD	Biochemical oxygen demand
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue R-250
COD	Chemical oxygen demand
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DSB	Direct scarlet B
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESI-MS	Electron spray ionization mass spectrometry
FPLC	Fast protein liquid chromatography
IAA	Iodoacetamide
KBA	Kiton blue A
Lcc1-8	Laccase isoforms (1-8)
LME	Lignin modifying enzymes
MEA	Malt extract agar
NCBI	National Centre for Biotechnology Information
O/N	Overnight
OP	Orange peelings

Abbreviations	Full forms
PDB	Protein data bank
RB5	Reactive black 5
RB21	Reactive blue 21
RBBR	Remazol brilliant blue R
RR198	Reactive red 198
RT	Room temperature
RV5	Reactive violet 5
SDS	Sodium dodecyl sulphate
TDS	Total dissolved solids
TEMED	N,N,N',N'-Tetramethylethylenediamine
TKN	Total kjeldahl nitrogen
TLC	Thin layer Chromatography
UV	Ultra violet
VA	Veratryl alcohol
WRF	White rot fungus/White rot fungi
WB	Wheat bran
WS	Wheat straw

LIST OF SYMBOLS

Symbols	Meanings
%	Percent
~	Approximately
bp	Base pair
^{o}C	Degree celsius
(μ/m) M	(Micro/ milli) Molar
(μ/m/n) g	(Micro/ milli/ nano) Gram
(m) l	(Milli) Litre
ε	Molar extinction coefficient
E^{o}	Redox potential
kDa	Kilo daltons
λ_{max}	Wavelength at which there is maximum absorption
OD	Optical density
ppm	Parts per million
rpm	Revolutions per minute
U	Enzyme activity unit
v/v	Volume/ volume
w/v	Weight/ volume

LIST OF ABBREVIATIONS FOR MICROORGANISMS

Microorganisms	**Abbreviation**
Cerrena unicolor	*C. unicolor*
Coprinus comatus	*C. comatus*
Coprinus cinereus	*C. cinereus*
Coprinopsis cinerea	*C. cinerea*
Cyathus bulleri	*C. bulleri*
Galerina marginata	*G. marginata*
Ganoderma lucidum	*G. lucidum*
Irpex lacteus	*I. Lacteus*
Laccaria bicolor	*L. bicolor*
Panus tigrinus	*P. tigrinus*
Phanerochaete chrysosporium	*P. chrysosporium*
Pichia pastoris	*P. pastoris*
Pleurotus florida	*P. florida*
Pleurotus ostreatus	*P. ostreatus*
Pycnoporus sanguineus	*P. sanguineus*
Stropharia aeruginosa	*S. aeruginosa*
Trametes gallica	*T. gallica*
Trametes hirsuta	*T. hirsuta*
Trametes pubescens	*T. pubescens*
Trametes trogii	*T. trogii*
Trametes versicolor	*T. versicolor*
Trametes villosa	*T. villosa*
Vigna radiata	*V. radiata*